A multiplex-system to target 16 male-specific and 15 autosomal genetic markers for orang-utans (genus: Pongo)

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TECHNICAL NOTE

A multiplex-system to target 16 male-specific and 15 autosomal genetic markers for orang-utans (genus: *Pongo*)

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Abstract Genetic studies of dispersal on local spatial and short temporal scales require a large number of autosomal microsatellites. However, the study of dispersal over large spatial scales and the resolution of deep evolutionary histories require marker systems that are preferentially inherited through the male or female line. Addressing such questions in endangered orang-utans (genus: *Pongo*) bears significant relevance to species conservation, as habitat destruction and fragmentation pose a significant threat to the whole genus. Here, we report 16 male-specific markers (nine human-derived microsatellites, six single nucleotide and one insertion-deletion polymorphisms), and 15 novel *Pongo*-derived autosomal microsatellite loci. All 31 markers can be amplified in four multiplex polymerase chain reactions even in DNA derived from faecal material. The markers can be applied to studying a wide range of important questions in this genus, such as conservation genetics, social structure, phylogeny and phylogeography.

Keywords *Pongo* spp. · Single nucleotide polymorphisms · Microsatellites · Y chromosome · SNP typing · Non-invasive samples

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The endangered orang-utans occur on the islands of Borneo (*Pongo pygmaeus*; about 50,000 animals) and Sumatra (*P. abelii*; about 6,500 animals), where they have undergone a recent dramatic decline in population size (Goossens et al. 2006; Wich et al. 2008). This has been mostly attributed to habitat loss, leading to heavily fragmented populations of often only a few hundred individuals (Wich et al. 2008). Therefore, it is essential to maintain genetic diversity, which has been linked to population fitness (e.g. Reed and Frankham 2003). This can be achieved by maintaining corridors between fragmented populations, allowing animals to follow natural dispersal patterns (Gilbert-Norton et al. 2010).

Studying natural dispersal in wild orang-utans pose significant challenges. Behavioural observations suggested higher male than female dispersal (Delgado and van Schaik 2000), although this has not been fully confirmed by previous genetic studies (Utami et al. 2002; Goossens et al. 2005), where patterns of direct dispersal were investigated using autosomal microsatellite markers. However, direct inferences from autosomal markers are limited to the timescale of a few generations and geographically small areas, as sexual recombination will break down sex-specific information (Goudet et al. 2002). Sex-biased dispersal over larger time and spatial scales can be investigated by contrasting genetic information obtained from markers inherited through either the male or female lineage (Handley and Perrin 2007).

In orang-utans, maternally transmitted mitochondrial DNA markers are widely available (e.g. Warren et al. 2001), but markers on the male-specific region of the Y chromosome have not yet been applied. Here, we report 16 male-specific markers for the application in the genus *Pongo*. Nine of these markers are human-derived microsatellite loci, six are single nucleotide polymorphisms (SNPs) and one is an

insertion-deletion polymorphism (indel). We also provide 15 novel orang-utan specific autosomal microsatellite markers, which yielded more reliable amplification results for use with highly degraded faecal samples than human-derived markers (data not shown).

In a previous study (Erler et al. 2004), 45 male-specific human-derived loci were shown to amplify in orang-utans. Of those, we selected 25 loci re-sequenced them, designed orang-utan specific primers, and tested for levels of polymorphism in 14 Bornean and Sumatran orang-utans (Table 1; see laboratory procedures below). After aligning flanking regions of all 25 microsatellite loci, we discovered two SNPs and one indel. Additionally, we identified four more SNPs by sequencing Y chromosome conserved anchored tagged sequence loci DBY13, SMCY12, and SMCY14 (Hellborg and Ellegren 2003). We combined 16 Y-specific polymorphic markers (GenBank Acc.No. HM803995-HM804006) into two multiplex PCRs (Table 1). Actual repeat number was linked to amplicon length through sequence data (Table S1 in the Online Resources). For SNP typing, we applied a modified fragment length discrepant allele specific PCR (Li et al. 2009). Each PCR combined a fluorescently labelled forward primer with two interrogating reverse primers. The reverse primers were complementary to one of each SNP allele at their 3′-end and differed in their length at the 5′-end; the resulting difference in product length allowed for allelic discrimination by fragment length analysis (Fig. 1a). One microsatellite locus had a SNP in close proximity and another a SNP and an indel, which allowed for simultaneous screening in one or two PCRs, respectively (Fig. 1). Multiplex reactions for Y markers consisted of 1 µl DNA template in an 8 µl volume, and were carried out with the Qiagen PCR Multiplex Kit according to manufacturer's instructions and varying primer concentrations (Table 1). In addition to the manufacturer's recommendations, we also included a touchdown step in the PCRs, starting at 4°C above the reported annealing temperature in Table 1, then decreasing by 0.5°C per cycle for eight cycles to reach the annealing temperature for the last 40 cycles. We assessed the variability of the Y-markers on a population level in 21 and 14 orang-utans from one Bornean (subspecies *P. pygmaeus wurmbii*) and one Sumatran site, respectively. In addition, we report the number of Y-polymorphisms in an extensive sample of 173 animals covering most of the range of the genus *Pongo* (Table 1, Nietlisbach 2009). We tested scoring reliability by repeating 32% of all genotyping reactions, and found an average scoring error rate of 0.6% over all loci. It has not escaped our notice that our results immediately suggest a possible diagnostic system for elucidating species affiliation or hybrid status for males of this genus

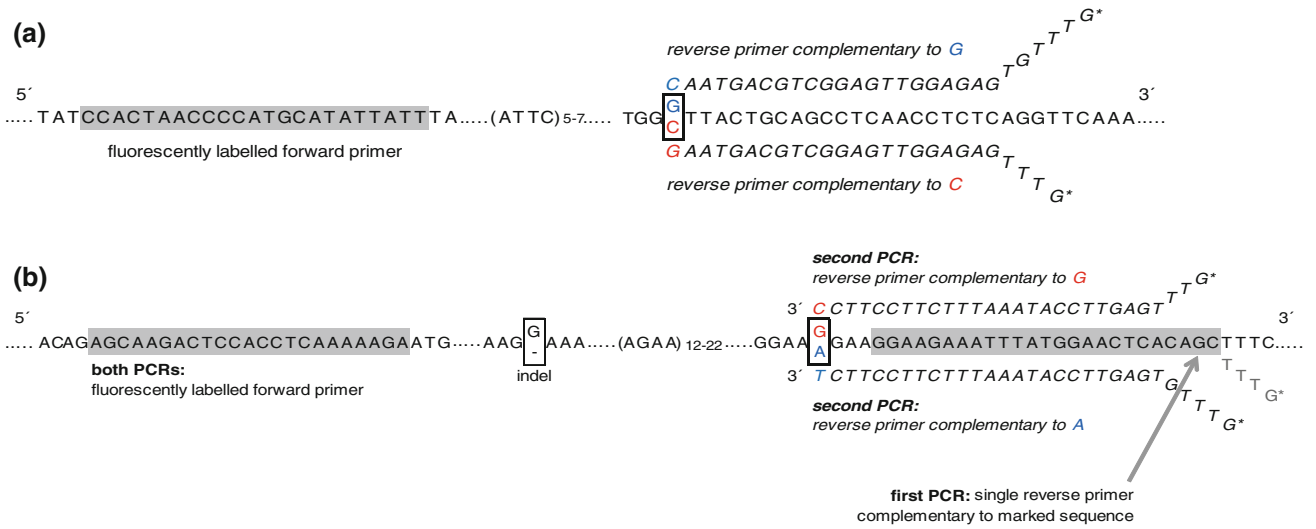

Fig. 1 Schematic representation of genotyping techniques for combined microsatellite/SNP typing. **a** Genotyping of SNP and microsatellite locus (DYS577). This locus was typed by a modified fragment length discrepant allele specific PCR (see text, Li et al. 2009) which targets the SNP in the box and the repeat motif of the microsatellite in one PCR. Discrimination between the two states of the SNP was achieved through competition of two reverse primers differing in length; and both reverse primers having a 1 to 2°C lower melting temperature and higher concentration than the fluorescently labelled forward primer (Table 1) to increase competition. Calibration was done with samples of known sequence. **b** Typing of three markers in two PCRs (DYS630). A first PCR using the grey primers amplified the region containing all three markers. A second PCR discriminates between SNP states as described in Fig. 1a. Due to confounding effects of the polymorphic microsatellite and indel, amplicon sizes of the first and second reactions have to be compared. If the amplicon of the first reaction is 5 bases longer than that of the second reaction, the SNP allele at this locus is G; if the difference is 3 bases, the SNP state is A. * PIG-tails were added to the 5′-end of reverse primers to enhance adenylation (Brownstein et al. 1996)

Table 1 Primers for male-specific and autosomal markers in orang-utans

Locus	Primer sequence 5′–3′	PC	T_A	Polymorphism	Overall		Suaq (Sumatra)				Tuanan (Borneo)			
					N	N_A	N	N_A	H_O	H_E	N	N_A	H_O	H_E
DYS630	*PET*-AGCAAGACTCCACCTCAAAAAGA*	0.15	63	AGAA	172	11	14	3		0.66	21	4		0.69
	gtttGCTGTGAGTTCCATAAATTTCTTCC	0.20		indel	172	2	14	1		0	21	1		0
DYS587	*6FAM*-AAAATTACCTTCTTTGGAAAGTAGTATT	0.30	63	ATACA	166	8	14	1		0	19	1		0
	gtttGTTATTTCTGAGCAGGGTTTCTAAG	0.40												
DYS532	*NED*-AGCAGGATTCCCTCTAAAAATATCA	0.10	63	compound,	171	3	14	1		0	21	2		0.09
	gTTTCTCCCTCCCTCCCTCTC	0.14		main motif (CTTT)										
DYS577	*6FAM*-CCACTAACCCCATGCATATTATT	0.30	63	GAAT	171	2	14	1		0	19	1		0
	gtttGAGAGGTTGAGGCTGCAGTAAG	0.40		C/G	171	2	14	2		0.13	19	1		0
	gtttgtGAGAGGTTGAGGCTG CAGTAAC	0.40												
DYS645	*6FAM*-GTACTAATTTTATTTCTTATGGCGTAGA	0.15	63	GTTTT	173	2	14	1		0	21	1		0
	gtttACACATGGCACCTGACACTG	0.20												
Y6C2	*6FAM*-CTTCTCCTTCTTCCTATTCTTCATCT	0.10	63	TTC	172	2	14	1		0	20	1		0
	gtttCAATAGTTTGGGGAAATAAGACAATG	0.14												
DBY13	*6FAM*-GGAAACTAAAATATGACATTGTAATTTG	0.30	63	C/G	168	2	14	1		0	20	1		0
	gtttATTTTTTTATTGTGATGCATACAGC	0.40												
	gtttgtATTTTTTTATTGTGATGCATACAGG	0.40												
DYS510	*PET*-GAAAGATAGATCAACAAGGTAGAAACAA	0.30	64	GATA	169	6	12	4		0.6	21	2		0.44
	gtttCATCCATCCATCCATCCATCT	0.40												
DYS561	*6FAM*-CCTGATGCCATCTGAAAATTAA	0.30	64	TAGA	168	5	14	1		0	20	3		0.52
	gtttACAACTGCACTCCAGCTTAGG	0.40												
DYS556	*6FAM*-TTACAAAACTAACATAAAGACCAAACAG	0.30	64	TAAA	172	3	14	1		0	21	2		0.41
	gtttGAAGCATTGAGTATAGTATAAAGTTGGT	0.40												
DYS630	*PET*-AGCAAGACTCCACCTCAAAAAGA*	0.15	64	A/G	171	2	14	1		0	21	1		0
	gttTGAGTTCCATAAATTTCTTCCTTCC	0.20												
	gtttgTGAGTTCCATAAATTTCTTCCTTCT	0.20												
SMCY12_26	*6FAM*-AAGGGTCACCACAGAAATACTTAG	0.15	64	C/G	173	2	14	2		0.13	21	1		0
	gtttgtCAGGTGGGGCGTAGTCTC	0.20												
	gtttCAGGTGGGGCGTAGTCTG	0.20												
SMCY12_337	*6FAM*-GTTACAGGTATACATGCACCTTTTT	0.15	64	A/C	171	2	14	1		0	21	1		0
	gtttgtGTGTGGCTTCTTTACTCTGTCA	0.20												
	gtttGTGTGGCTTCTTTACTCTGTCC	0.20												
SMCY14	*6FAM*-ATGGGGAAAAGATGAGTTCTGA	0.15	64	C/T	173	2	14	1		0	21	2		0.41
	gtttGTCTGGCATCCTAATGCCT	0.20												
	gtttgtGTCTGGCATCCTAATGCCC	0.20												

Table 1 continued

Locus	Primer sequence 5′–3′	PC	T_A	Polymorphism	Overall		Suaq (Sumatra)				Tuanan (Borneo)			
					N	N_A	N	N_A	H_O	H_E	N	N_A	H_O	H_E
O4_6	*PET*-GGCAATGTAACATATCCCTCTGTGT	0.05	58	GATA			23	4	0.61	0.68	28	3	0.71	0.62
	AGCCATGGACCTTGTGAGAAAAG	0.05												
O4_A5	*6FAM*-ATGGGCCCAGAAAACAACTCAGT	0.15	58	(GATA)(GATG)			22	4	0.64	0.55	26	6	0.65	0.65
	AGATAAAGGAATGGATAGATGGACAGA	0.15												
O4_A7	*VIC*-ACTGGCCCATTCAAAGTCTGTCATT	0.10	58	GTAG			21	4	0.86	0.72	26	2	0.35	0.29
	ACTGGCCCATTCAAAGTCTGT	0.10												
O4_A8	*NED*-CACAGGGTCCAAACTCAGATTATTG	0.20	58	(GATA)(GATG)			23	3	0.30	0.31	29	1	0	0
	CCTTCCCCCTCATGTAGTTATCAA	0.20												
O4_B5	*VIC*-GAGCCCTGATTCGTTTTACTGG	0.20	58	GATA			22	6	0.86	0.73	28	5	0.50	0.54
	AGCAAAGGCAGAAAACTGTAATGA	0.20												
O4_B6	*6FAM*-TGGAGCCTGAATATGTGACTGAAT	0.20	58	(GATA)(GTAG)			20	6	0.65	0.61	26	6	0.46	0.79
	AATGCCAGGATTTCCTTCTTTTT	0.20												
O4_B24	*6FAM*-TCTGAGGTACCCTGTAACAAAGAAA	0.10	58	GATA			23	3	0.65	0.56	29	1	0.00	0.00
	GAAATCCCAGTACCATATAAATGTCAT	0.10												
O4_A1	*6FAM*-CTCCCCTTCCTTCCTTTATTCAGTT	0.10	62	GTAG			23	5	0.87	0.73	28	4	0.82	0.75
	CAACACTTGGCAGTCACAAATCAG	0.10												
O4_B3	*VIC*-TTCCAGAAGGGGCGAGAAGTT	0.10	62	GACA			22	3	0.59	0.64	26	1	0	0
	GTTGGGACCAAACAGTTGTCAATAA	0.10												
O4_B17	*PET*-GTACCGACGGTGCACGAACAATGTA	0.30	62	GATG			19	3	0.68	0.67	26	6	0.69	0.73
	AGCCTGGCTGAAAAGTGGAACTGAG	0.30												
O4_B20	*NED*-CCTGCATTTTGTCACTCCCTCAACC	0.20	62	GATG			14	1	0	0	24	2	0.33	0.38
	CTGCCACACCTCCATGGACACAGAT	0.20												
O4_C9	*6FAM*-TGCAGGCCAGGGCTTCTTTCAA	0.15	62	GATA			22	5	0.55	0.54	27	4	0.59	0.63
	CAGTCTCCCCAGGACCCCTACACAG	0.15												
O4_C13	*6FAM*-CTGGGCACACTGTATATGGGGTAG	0.20	62	GATA			20	3	0.75	0.56	21	4	0.38	0.59
	GTTTGAGACCACTCATGATGCAAAGACC	0.20												
O4_Chr5	*PET*-CAGCAGCTCCTGAAATATCTGTCC	0.15	62	GATA			21	4	0.81	0.70	23	5	0.74	0.74
	GTTTGGGGTAGAGGAAAGCAGGTTGAT	0.15												
O4_Chr7	*NED*-CATCTCTTTATGGCTGACTGTTGAT	0.10	62	GATA			17	11	0.76	0.83	24	15	0.92	0.91
	GTTTGGTCCAAGACAAATTTGTATGAGT	0.10												

All loci with a Y in the name are Y-linked, all others are autosomal. Summary statistics are given for two study sites and over all sampled orang-utans. For loci DYS630 and DYS577, three and two male-specific markers were typed, respectively (Fig. 1). Loci combined in a single multiplex reaction have the same annealing temperature

Fluorescent labels are shown in italics at the 5′ end of the forward primer. PIG-tail bases (Brownstein et al. 1996) are given in lower case

PC primer concentration [μM], T_A annealing temperature, *N* number of samples, N_A number of different alleles, H_O observed heterozygosity, H_E expected heterozygosity (Nei 1987). * primer used in two PCRs (Fig. 1b)

Amplicon sizes and their relation to repeat numbers are shown in Table S1 in the Online Resources

(Nietlisbach 2009), if used in unison with readily available mtDNA markers.

To clone autosomal microsatellite markers, we extracted genomic DNA from 25 mg of frozen muscle tissue from a Sumatran orang-utan, using the DNeasy Tissue Kit (Qiagen). We digested ten micrograms of the purified DNA with *Nhe*I and *Alu*I (New England Biolabs) and size-selected for fragments between 400 and 1,200 base pairs length. Enrichment, cloning and sequencing were carried out as described in Nater et al. (2008), using only tetranucleotide biotinylated probes [$(GACA)_7$, $(GATA)_7$, and $(GATC)_7$]. We sequenced plasmids from 68 positive clones, of which 70% contained a microsatellite repeat. For 25 loci, which contained long uninterrupted repeats, we designed primers and amplified these loci in twelve orang-utans. Levels of polymorphism were qualitatively assessed on high-resolution Spreadex gels (Elchrom Scientific). Based on these results, we fluorescently labelled the forward primers of the 15 most polymorphic markers and combined these 15 loci (GenBank Acc.No. HM804007-HM804021) into two multiplex PCRs (Table 1). Then, we genotyped 29 orang-utans from Borneo and 23 from Sumatra, using DNA extracts from faecal samples with target DNA concentration ranging from 25 to 1,000 pg/µl, strictly following guidelines from Morin et al. (2001). PCRs using the Qiagen PCR Multiplex Kit contained 1 µl template DNA in an 8 µl final volume, with varying primer concentrations and annealing temperatures (Table 1). PCRs included 45 cycles with conditions according to manufacturer's instructions.

If not indicated otherwise, we used standard laboratory techniques at each step. We designed PCR primers with the PrimerSelect software implemented in Lasergene v7 (DNASTAR). PCR amplifications were performed on Veriti 96-well thermal cyclers (Applied Biosystems). Sequencing reactions were carried out using the BigDye Terminator v3.1 on a 3730 DNA Analyzer (both Applied Biosystems) according to manufacturer's instructions, cleaned-up using a $MgSO_4$ precipitation procedure, followed by resuspending the pellet in 20 µl ddH_2O. For fragment length analysis, PCR products were diluted 20–80 times in ddH_2O. One microlitre of this was added to 9.93 µl HiDi formamide and 0.07 µl of GeneScan 500 LIZ Size Standard (both Applied Biosystems) and denatured for three minutes at 95°C. We ran the samples on a 3730 DNA Analyzer and obtained genotypes using GeneMapper software v4.0 (Applied Biosystems). For the statistical analyses, we used MStools v3.1 add-into Microsoft Excel (Park 2001) and Genepop v4.0 (Rousset 2008).

Fragment length discrepant allele specific PCR used as SNP typing technique proved to be a reliable and cost-efficient strategy to assess SNP variation. The possibility to combine this technique with conventional microsatellite fragment length analysis makes it a suitable method to include a small number of SNPs to complement an extensive microsatellite analysis. The polymorphic male-specific markers for orang-utans described here promise to be highly useful for population genetic and phylogenetic studies addressing questions about dispersal strategies, phylogeographic patterns, and comparisons with other molecular markers. The autosomal markers can be applied to investigate local dispersal or assess relatedness and paternity. Knowledge about such processes, in particular about natural dispersal strategies, is important for species conservation.

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